

The Effect of Strontium on Embryonic Calcification of *Aplysia californica*

JOSEPH P. BIDWELL¹, ALAN KUZIRIAN², GLENN JONES³,
LLOYD NADEAU⁴, AND LISA GARLAND³

*Howard Hughes Medical Institute, Woods Hole Oceanographic Institution,
Woods Hole, Massachusetts 02543*

Abstract. During embryogenesis of the marine opisthobranch gastropod *Aplysia californica* Cooper, 1863, there is a brief critical time (window) during which strontium is essential for the onset of calcification. The present study was undertaken to elucidate the role of this element in mineralization. Strontium performed no structural function; deformed shells of strontium-deprived animals had normal atomic crystal structure and the element was excluded during calcification. Calcium deposition and fixation was reduced by approximately 80% in the absence of strontium but was not significantly altered in the presence of sub-optimal concentrations of this metal ion despite dramatic deficits in shell and statolith morphology. This suggests that calcium deficiency *per se* is not responsible for deficits induced by strontium deprivation. The reduced total calcium may be a secondary effect resulting from the complete inhibition of precipitation. Strontium did not modulate total alkaline phosphatase activity or total sulfated mucopolysaccharide synthesis during embryogenesis, and no morphological abnormalities of the organic shell were observed. Although the role of strontium in embryonic calcification of *Aplysia californica* remains enigmatic, these data suggest that strontium affects a highly discrete regulatory component because these more general indicators of

calcification and differentiation are unaffected by its absence.

Introduction

Strontium is required for the normal embryonic development of a variety of marine molluscs including gastropods (Bidwell *et al.*, 1986), bivalves (Gallager *et al.*, 1989), and cephalopods (Hanlon *et al.*, 1989). Embryos reared in the absence of strontium lack mineralized statoliths or shell, yet the soft-tissues appear normal. There is a critical window for strontium during embryogenesis of the opisthobranch gastropod *Aplysia californica* Cooper, 1863; normal mineralization requires 4 ppm strontium [$\sim 45.7 \mu\text{M}$, half the concentration of natural seawater (Bruland, 1983)], lower concentrations (1–3 ppm) or exposure to high levels (80 ppm) results in abnormal and incomplete calcification (Bidwell *et al.*, 1986).

The dramatic specificity of the effect of strontium on molluscan embryogenesis affords a unique opportunity to study mineralization, a process only superficially characterized for any organism (Krampitz and Graser, 1988). Is the role of strontium primarily structural, as in the SrSO_4 tests of the marine protozoa *Acantharia cantharia* spp. (Anderson, 1981), or is a biochemical mechanism operative?

We report here the effects of varying the strontium concentration of artificial seawater on the atomic crystal structure of the embryonic shell and statoliths of *Aplysia californica*. We also describe the effects of medium strontium concentration on whole embryo levels of calcium, strontium, and magnesium throughout embryogenesis. Finally, we present histochemical analyses of strontium's influence on alkaline phosphatase activity and mucopolysaccharide synthesis.

Received 19 July 1989; accepted 26 February 1990.

¹ Present address: Endocrine Research Unit, Mayo Clinic and Foundation, Rochester, Minnesota 55905. To whom requests for reprints and all correspondence should be addressed.

² Marine Biological Laboratory, Woods Hole, Massachusetts 02543.

³ Woods Hole Oceanographic Institution.

⁴ Present address: Toxikon, 225 Wildwood Avenue, Woburn, Massachusetts 01801.

Materials and Methods

Bioassay

Bioassays were conducted as described previously (Bidwell *et al.*, 1986). Briefly, a fresh egg mass of *Aplysia californica* was washed in basal medium, an artificial seawater consisting of the major salts of natural seawater except $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$. Stock solutions of $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ were standardized via flame atomic absorption. The egg mass was cut into strands and distributed among treatments. Natural seawater (NSW) was prefiltered ($\sim 1 \mu\text{m}$, Millipore) before use and the pH and salinity recorded (pH ~ 8.0 , salinity 32‰). All experiments were conducted at 21–22°C under constant illumination. Treatment waters were replaced with fresh media as noted below.

X-ray crystallographic experiments

Embryos were reared in one of three treatments including basal medium plus 3 ppm Sr (strontium, 34.2 μM),⁵ basal medium plus 80 ppm Sr (0.913 mM) and NSW. To obtain sufficient material for X-ray analysis, approximately 30 g of egg strands were maintained in 12 l of each test medium. Media were aerated constantly and replenished daily.

At 168 h after oviposition, strands were removed from treatment, rinsed with a 4% solution of ammonium acetate (to pH 8 with ammonium hydroxide), stored at -80°C until lyophilized, soaked in 5% reagent sodium hypochlorite (Baker) for 24 h, and the empty shells rinsed and dried with methanol. Adult shells, removed from the mantle tissue of 20 animals, were prepared as described above.

Samples were analyzed for crystal structure using X-ray diffraction methods. One-quarter to one-half gram of clean shells was backed with 40 mesh granular zinc and press-mounted onto standard Phillips aluminum sample holders using an applied pressure of 2 tons. Data were obtained with a Phillips Model 3500 XRD with a fine-focus Cu-K α X-ray tube operated at 40 kV and 20 ma. A theta compensator slit was placed between the sample and the X-ray tube, and a 0.2° receiving slit and graphite crystal monochromator were placed between the sample and the scintillation detector.

The X-ray analysis was controlled using an IBM-AT computer and all data were collected and stored digitally.

⁵ We express medium ionic concentrations initially in units of parts per million (ppm) and molar (M) but use ppm thereafter. Whole embryo concentrations of calcium, strontium and magnesium are expressed in moles/mg ash because mole units are more descriptive when comparing concentrations of different elements (see Bidwell and Spotte, 1985, for a review of this topic).

Each sample was scanned from 3 to 60° 2 θ (29.45–1.54 Å d-spacing) in 0.03° steps, counting for 3 s between each step.

Calcium, strontium, and magnesium analysis of whole embryos

Egg strands (1 cm) from a single egg mass were distributed randomly between the five treatments of basal medium (0 ppm Sr), 3 ppm Sr, basal medium plus 8 ppm Sr (91.3 μM), 80 ppm Sr, and NSW. Three egg strands were placed in each covered petri dish and its replicate. The test media (50 ml/dish) were replaced twice daily. Throughout development, six strands were collected for each treatment, rinsed with the ammonium acetate solution, transferred to microcentrifuge tubes (1.5 ml), plunged into liquid nitrogen, and stored at -80°C . Egg strands were lyophilized, the individual dry weights recorded, and ashed at 500°C (3 h) for ash weight. The ashed strand was digested to dryness with 50 μl of concentrated nitric acid (EM, Specpure) and stored at 4°C. One hour before analysis the residue was dissolved in 0.1 N HCl (EM Specpure)/5 mM $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ (Fisher AA grade).

A Perkin Elmer 2280 atomic absorption spectrophotometer equipped with an HGA-400 graphite furnace was used to determine total calcium, magnesium, and strontium [methodology after Delaney (1983)] in whole embryos. Calcium and magnesium were analyzed via flame spectroscopy and strontium was determined with the furnace.

The strontium and magnesium distribution coefficients were determined from these data. The distribution coefficient is defined as:

$$K_M = \frac{[M]/[\text{Ca}] \text{ tissue}}{[M]/[\text{Ca}] \text{ medium}}$$

where M is the mole concentration of strontium or magnesium (Dodd, 1967). This ratio is a unitless index for characterizing biochemical and/or crystallographic discernment. A value of one indicates a lack of regulation, below one is evidence for discrimination and above one concentration. In this study the $[M]/[\text{Ca}]$ ratio of the whole embryonic tissue, including the egg strand, was determined rather than the tissue or mineral alone because a clean separation of the embryo, shell, statolith, and egg strand with ultrapure reagents was not successful.

Alkaline phosphatase experiments

Egg strands, from a single egg mass, were maintained either in 2-l erlenmeyer flasks under constant aeration (45, 1 cm strands/flask) or in the petri dishes (3, 1 cm

strands/dish). Test media were replenished every 48 h for the 2-l flasks and twice daily for the petri dishes. Treatments included NSW, 0 ppm Sr, 8 ppm Sr, and 8 ppm Sr plus 0.09 ppm beryllium (10 μ M Be, as BeSO₄·4H₂O). Be is a potent inhibitor of alkaline phosphatase, both *in vitro* (Aldridge, 1950; Chevremont and Firket, 1951) and *in vivo* (O'Day, 1972). Dose response experiments were conducted with Be (9×10^{-5} to 45 ppm) in the presence of 8 ppm strontium.

Histochemistry. Both the histochemical and biochemical assays for alkaline phosphatase were developed in the laboratory of Richard Whittaker, Marine Biological Laboratory, and kindly made available to us. Embryos were relaxed, while encapsulated, by the addition of 8% MgCl₂·6H₂O to the test medium. Egg strands (3–4 mm) were prefixed with 4% formaldehyde (from paraformaldehyde) in 0.2 μ m filtered NSW for 30 min (4°C). The strands were incubated in 10 ml of Tris-buffer solution (100 μ M Tris-Base, 100 mM NaCl, 5 mM MgCl₂·6H₂O, pH 9.5) containing 33 μ l of nitro blue tetrazolium (Sigma), (50 mg/ml in 70% dimethylformamide) (Sigma), and 33 μ l of 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (Sigma), (25 mg/ml in 100% dimethylformamide). The color reaction was monitored over 30–60 min with a dissecting scope (21°C). Fixation was continued with 3% glutaraldehyde/1.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 5 mM EGTA, 5 mM MgCl₂·6H₂O, and 10% sucrose (4 h to overnight).

Whole mounts were prepared by transferring the egg strands from the cacodylate/sucrose buffer to 0.1 M sodium cacodylate and cutting the capsules to free the embryos. Specimens were rinsed with 50% methanol, dehydrated with dimethoxypropane (DMP, Muller and Jacks, 1975), further rinsed with 100% ethanol, cleared with Histoclear (National Diagnostics, Sommerville, New Jersey) and mounted on a glass slide using Diatex (Scientific Products).

For tissue sections, egg strands were post-fixed in 1% osmium in 0.1 M cacodylate (40–45 min, 21°C), rinsed 3 \times with 0.1 M cacodylate followed by 50% methanol, and dehydrated in DMP. Following transfer to propylene oxide, the tissues were infiltrated and embedded in Epon/Araldite. The sections were cut (2 μ m), heat mounted on clean, untreated slides, and epoxy extracted with a solution of saturated KOH in 100% ethanol for 10 min. These sections were cleared and mounted to check the specific location of the reaction product. Sections and whole mounts were examined using a Zeiss Universal microscope equipped with a polarizing and differential interference contrast (DIC) optics.

Biochemical analysis. Egg strands were transferred to microcentrifuge tubes, plunged into liquid nitrogen, and

stored at -80°C until analysis. Samples were thawed on ice and homogenized for 1 h in lysis buffer (4°C) containing 50 mM Tris HCl, 1 mM MgCl₂·6H₂O, 1 μ M ZnCl₂, 0.025% (w/v) Triton-X, and 0.0625% (w/v) sodium deoxycholate. The homogenate was centrifuged at $25,000 \times g$ for 10 min, 4°C. Alkaline phosphatase activity and total protein of the supernatant were determined colorimetrically via the p-nitrophenol (Sigma) and Bradford (Biorad, microassay) methods, respectively. Modifications of the alkaline phosphatase method included an extended incubation time for color development (16 h vs. 15 min) and a lower incubation temperature (21°C vs. 37°C, Whittaker, pers. comm.). Alkaline phosphatase activity was normalized to total protein.

SEM analysis. Embryos from Be dose-response experiments were processed for scanning electron microscopy (SEM) as described previously (Gallagher *et al.*, 1989).

Mucopolysaccharide synthesis experiments

Egg strands were maintained in 2-l flasks or petri dishes as described above. Treatments included 0 ppm Sr, 8 ppm Sr, and NSW. Samples were removed from the treatments throughout embryogenesis and analyzed for mucopolysaccharides.

Histochemistry. Following experimental treatment the encapsulated embryos were relaxed with 8% MgCl₂·6H₂O and transferred to the complete glutaraldehyde/paraformaldehyde fixative described above supplemented with 0.1% ruthenium red. Fixation lasted 2–4 h (20°C) or overnight (4°C) followed by rinsing and storage in 0.1 M cacodylate buffer plus 25% sucrose. Following routine methods outlined above, the embryos were embedded in epoxy resin. Tissue sections were cut, heat mounted on slides, epoxy extracted and treated with the following stains: Aldehyde fuchsin/alcian blue (pH 2.5) in sequence (Spicer and Meyer, 1960); 0.5% alcian blue (pH 2.5) alone, and 1% alcian blue (pH 1.0) (Lev and Spicer, 1964).

Statistical analysis

Values were log transformed for total calcium, strontium, and magnesium concentrations and alkaline phosphatase-specific activities. A two-way ANOVA was used to compare data from two or more treatments; time and treatment were fixed factors and the significance of the time \times treatment interaction was determined. Means and least-square means were compared followed by the Student-Newman-Keuls (SNK) multiple comparison test and pairwise contrasts (adjusted by the Bonferonni method), respectively. The calcium and magnesium concentrations for the 0 ppm Sr treatment were analyzed

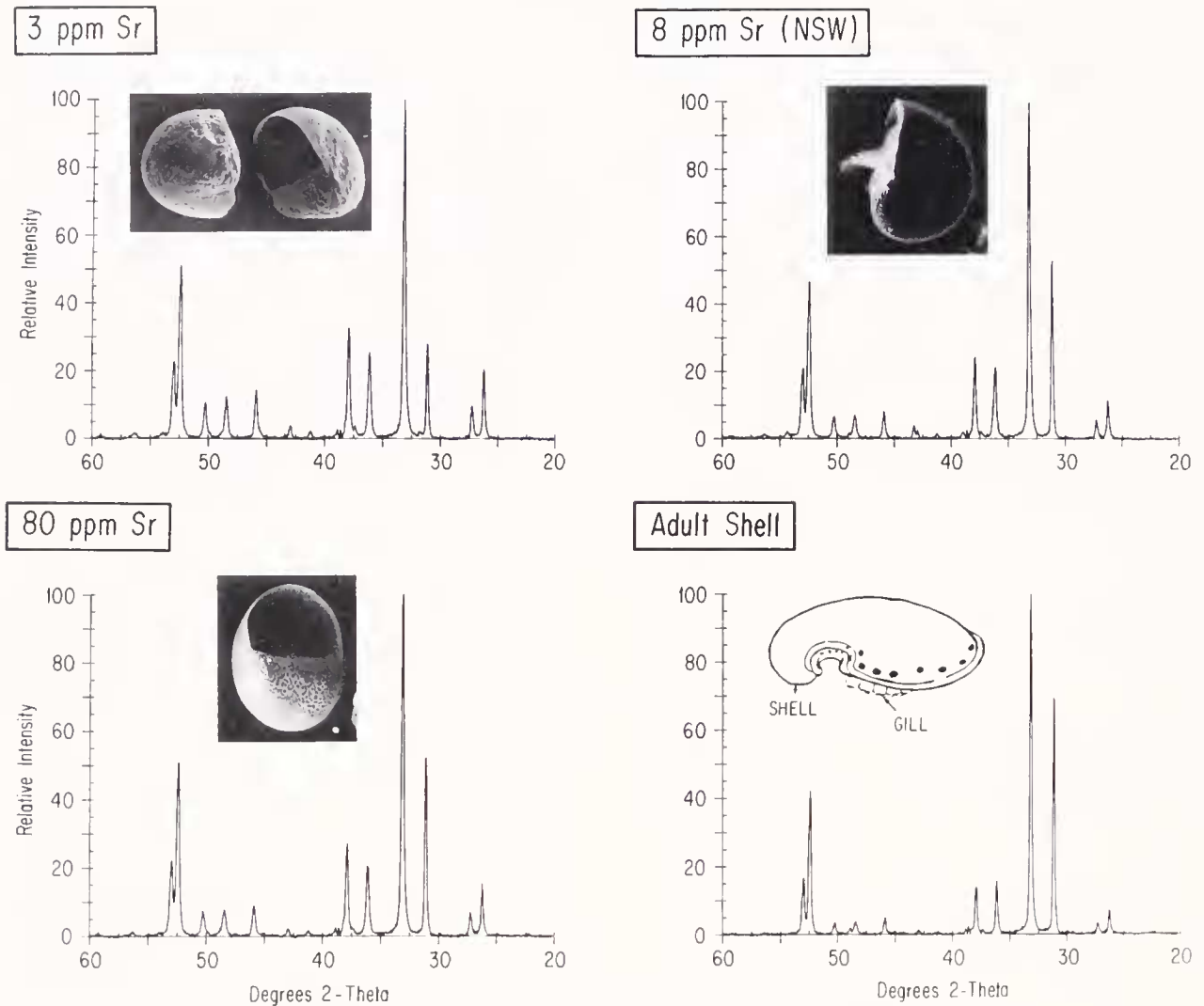


Figure 1. X-ray diffraction spectra of mineralized shell and statolith from indicated treatments. All material was aragonite and there was no evidence of paracrystalline or amorphous material in the deformed shells (3 and 80 ppm Sr).

separately using a one-way ANOVA followed by the SNK. Calculations were performed with SAS.

Results

X-ray crystal structure

Atomic diffraction spectra of abnormal shells and statoliths from embryos reared in the 3 and 80 ppm Sr media were indistinguishable from spectra of normal tissue (Fig. 1). All diffraction peaks observed for each sample were identified as belonging to the aragonite form of calcium carbonate. There were no significant shifts in peak position, changes in peak heights, nor evidence of amorphous or paracrystalline structure in the abnormal mineralized tissue.

Whole embryo concentrations of calcium, strontium, and magnesium

Total calcium at the end of embryogenesis (192 h) was diminished by approximately 80% in those embryos reared in the absence of strontium as compared to those organisms from strontium-containing media; total calcium of embryos from the 3 and 80 ppm Sr treatments were not significantly different from levels of the control organisms (8 ppm Sr, NSW, Fig. 2A). Thus, no correlation between total calcium and shell morphology was evident. Profiles for calcium concentration paralleled mineralization in all media containing strontium (Fig. 2A). At 96 h, a small but statistically significant increase in total calcium was observed coincident with the appear-

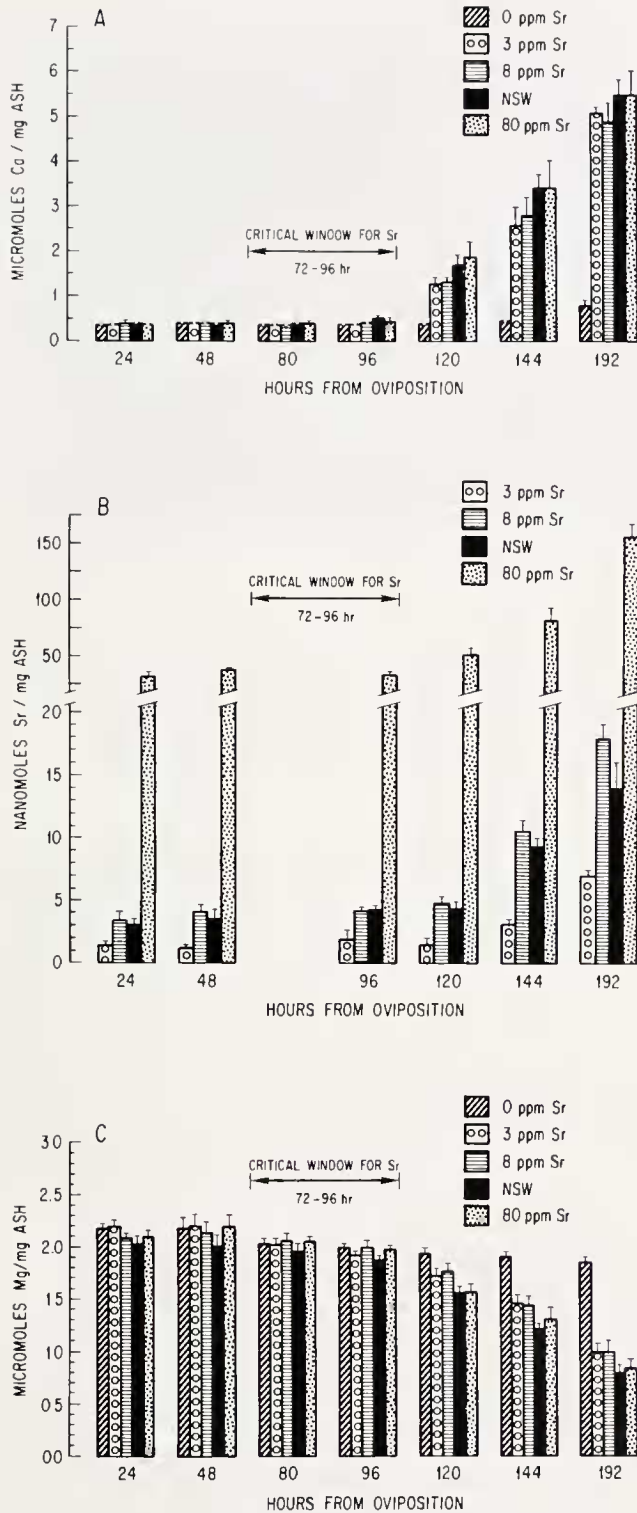


Figure 2. Chemical analysis of whole embryos plus egg strand. (A) Total calcium; a small but statistically significant increase was observed at 96 h (total calcium at 24–80 h = $0.39 \pm 0.02 \mu\text{M}/\text{mg ash}$; total calcium at 96 h = 0.45 ± 0.05 , mean of combined treatments 3, 8, 80 ppm Sr and NSW \pm S.D., $P < 0.05$). There was no significant difference in total calcium levels nor a significant time \times treatment interaction be-

ance of the shell cap and statolith granules. Total calcium was not observed to increase in animals from the basal medium until 144 h, without evidence of mineralization.

Profiles representing total strontium concentration of whole embryos paralleled those for calcium but were proportional to medium levels (Fig. 2B).

Total magnesium concentration of whole embryo decreased during embryogenesis and this decline was attenuated in the absence of strontium (Fig. 2C). Although small, the decrease in whole embryo magnesium concentration over time from the 0 ppm Sr treatment was significant ($P < 0.001$).

Comparison of the strontium distribution coefficients (K_{Sr}) over time indicated that the discriminatory mechanism for this element was not acquired until after the critical window. K_{Sr} (Fig. 3A) for all treatments containing strontium had values close to one before the onset of mineralization, (1.05 ± 0.01 , mean \pm S.E., combined means of all treatments containing strontium, 24–96 h, $n = 12$). An abrupt decrease in the values of K_{Sr} (0.34 ± 0.03 , mean \pm S.E., $n = 4$) was observed at 120 h as mineralization began. The distribution coefficient for synthetic aragonite prepared from artificial seawater is approximately 1.0 at room temperature (Kinsman, 1969).

The values of the magnesium distribution coefficient, K_{Mg} , for the 0 ppm Sr treatment ranged from 1.16 ± 0.02 at 24 h to 0.45 ± 0.07 at 192 h (mean \pm S.D., $n = 5$ and 6, respectively) indicating discrimination of this element (Fig. 3B). This may represent the biochemical component of the regulatory mechanism for total magnesium because shell formation was absent.

Alkaline phosphatase activity

Positive staining for alkaline phosphatase in whole specimens was coincident with the onset of the critical window (approximately 72 h) and was localized in areas where mineralization was imminent, *i.e.*, the prevelar lobes (statoliths) and the shell field; intensity of the stain was not dependent upon whether the organism had been

tween the 3 ppm and 8 ppm profiles or the NSW and 80 ppm profiles. Planned pairwise comparisons between the ASW treatments 3, 8, and 80 ppm Sr revealed that differences in calcium concentrations were limited to 120 and 144 h ($P < 0.001$). (B) Total strontium; this element was not detected with graphite furnace analysis in organisms from the 0 ppm Sr medium. Strontium samples (80 h) were unsuitable for analysis from this particular experiment. (C) Total magnesium; there was no significant difference between the 3 and 8 ppm Sr profiles but a significant contrast between these two profiles and that for the 80 ppm Sr treatment from 120 h through 192 h ($P < 0.001$). There was also a significant difference between the 80 ppm Sr and NSW profiles ($P = 0.003$) but no difference in magnesium levels at 192 h.

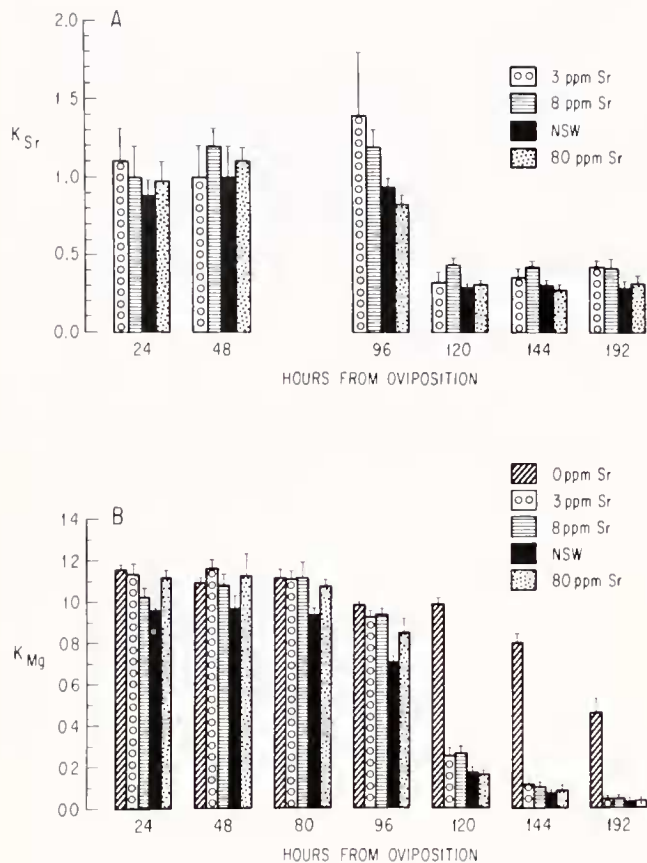


Figure 3. Distribution coefficients for (A) strontium and (B) magnesium for indicated treatments (mean \pm S.D., $n = 3-6$).

reared in the presence of strontium (0 and 8 ppm Sr, Figs. 4A, B). Staining intensified in both treatments as development progressed, encompassing the velum, mantle, and the statoliths (8 ppm Sr) or the empty statocyst cavities (0 ppm Sr).

Exposure to beryllium (0.009–0.09 ppm), a potent inhibitor of alkaline phosphatase (Aldridge, 1950; O'Day, 1972), resulted in abnormalities of the embryonic shell and statoliths similar to those observed for strontium-deprived organisms (Fig. 4C). Nevertheless, this metal ion (0.09 ppm) did not attenuate alkaline phosphatase activity as measured biochemically (Fig. 4D), nor was the measured activity diminished in organisms from 0 ppm Sr.

Mucopolysaccharide synthesis

The presence of strontium had no effect on mucopolysaccharide synthesis as indicated by histochemical analysis, despite the failure of the organic shell to tan in those animals from the basal medium. Whole specimens from both the 0 and 8 ppm Sr treatments stained darkly with

ruthenium red during embryogenesis; the indicator appeared evenly dispersed throughout the soft tissue, the organic matrix, and the egg strand. Differential staining with alcian blue and aldehyde fuchsin demonstrated the presence of highly sulphated mucopolysaccharides and an absence of the acidic forms; again no differences were detected in embryos from the 0 and 8 ppm Sr treatments. No morphological abnormalities of the organic shell of embryos from the 0 ppm Sr treatment were observed upon examination of thick sections using light microscopy.

Discussion

The marine protozoa *Acantharia spp.*, the only other organisms known to require strontium, use the element for the formation of their $SrSO_4$ tests (Anderson, 1981). Paradoxically, the deficits as a result of strontium deprivation of *Aplysia californica* are specific for calcification, yet the defect is not mineralogical. We have demonstrated that strontium is discriminated against during mineralization and is not required for the stabilization of the aragonite polymorph in seawater. This element prevents the aragonite-to-calcite transition in calcium carbonate preparations (McLester *et al.*, 1970; Yoshioka *et al.*, 1986).

Because strontium is not part of the shell itself, this element may regulate a biochemical pathway vital to the onset of mineralization. Total embryonic calcium was reduced dramatically ($\sim 80\%$) in organisms reared in the absence of strontium, but calcium increase was not abolished. Furthermore, there was no correlation between total calcium levels at the end of embryogenesis and shell morphology in organisms from strontium-containing media. This suggests that calcium deficiency *per se* is not responsible for deficits induced by strontium deprivation. The reduced calcium may be a secondary effect resulting from the complete inhibition of precipitation. The present data do not address whether calcium uptake or transport is modulated by strontium and isotope experiments will be required to investigate this possibility.

Alkaline phosphatase has been implicated as a nucleating agent in precalcifying matrices (Vittur *et al.*, 1984; Marks and Popoff, 1988), and, although the onset of alkaline phosphatase activity was coincident with the critical window, it was not dependent upon the presence of strontium. Beryllium did not diminish this activity, although its presence during embryogenesis resulted in defects remarkably similar to those observed as a consequence of strontium's absence. Beryllium's inhibitory action on alkaline phosphatase is immediately reversible with magnesium in both histo- and biochemical preparations (Raven, 1966; Aldridge, 1950), and therefore mag-

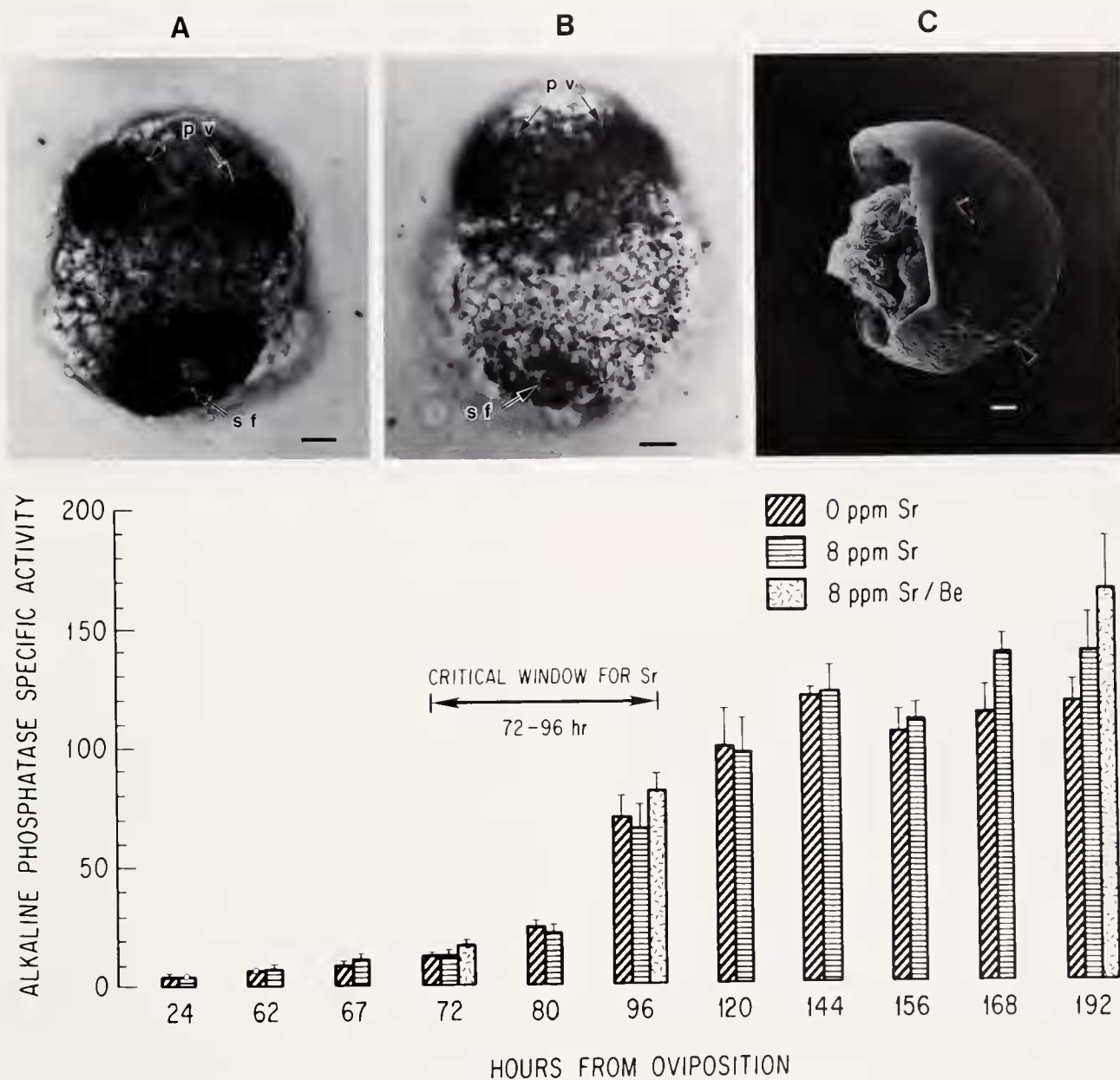


Figure 4. Alkaline phosphatase analysis. (A) Light micrograph showing dark histochemical staining for alkaline phosphatase activity in the, prevelar lobes (pv), and shell field (sf) of *Aplysia californica* embryo, 72 h, from ASW + 8 ppm Sr. (B) as in (A) for 72 h embryo from ASW without Sr. (C) SEM micrograph showing effect of 0.09 ppm Be on shell formation of embryonic *Aplysia californica*, shells were malformed and crenulated (arrowheads). (scales = 10 μ m). (Graph) Specific alkaline phosphatase activity (normalized to total protein) of animals from the indicated treatments (mean \pm S.D., n = 3). Only three time points were collected and analyzed for the 8 ppm Sr/0.09 ppm Be treatment. The largest increment in total alkaline phosphatase specific activity as measured biochemically was observed during the critical window in organisms from both the 0 and 8 ppm Sr treatments.

nesium may substitute for strontium *in vitro* although the stringent specificity for the latter *in vivo* argues against this possibility.

A defect in the organic matrix could severely limit or

inhibit nucleation, but we were unable to demonstrate any morphological abnormalities in the organic shell nor a diminution of sulfated mucopolysaccharide synthesis in those embryos reared in the absence of strontium. The

molluscan matrix is not well characterized and our results do not preclude the formation of other matrix components being strontium-dependent.

Strontium's role and mechanism of action remain enigmatic. While essential for shell formation, the element performs no structural function. Moreover, our preliminary data suggest that strontium affects a highly discrete regulatory component because more general indicators of calcification and embryogenesis, *e.g.*, alkaline phosphatase activity, sulfated mucopolysaccharide synthesis, and soft tissue differentiation, are seemingly unaffected by its absence. Continued histo- and biochemical screening for a strontium-dependent marker will be an appropriate component of future work. Candidates for investigation will include acid phosphatases, Ca^{2+} -ATPase, and lysosomal acid hydrolases—all enzymes associated frequently with mineralization (Linde, 1981). Elucidation of the importance of strontium to seashell formation should contribute to our understanding of the biomineralization processes.

Acknowledgments

We thank Drs. James H. Schwartz and Eric R. Kandel (Columbia University, the Howard Hughes Medical Institute) for their support throughout this research. Donna McPhie (Marine Biological Laboratory) assisted with the histochemical studies. Scott Gallagher of the Woods Hole Oceanographic Institution read the manuscript critically and contributed helpful suggestions throughout the work.

Literature Cited

- Aldridge, W. N. 1950. Beryllium and alkaline phosphatase. *Nature* **165**: 772.
- Anderson, O. R. 1981. Radiolarian fine structure and silica deposition. Pp. 347–379 in *Silicon and Siliceous Structure in Biological Systems*, T. L. Simpson and B. E. Volvanti, eds. Springer-Verlag, New York.
- Bidwell, J. P., and S. Spotte 1985. *Artificial Seawaters. Formulas and Methods*. Jones & Bartlett, Inc. Boston. 349 pp.
- Bidwell, J. P., J. A. Paige, and A. M. Kuzirian. 1986. Effects of strontium on the embryonic development of *Aplysia californica*. *Biol. Bull.* **170**: 75–90.
- Bruland, K. W. 1983. Trace elements in seawater. Pp. 157–220 in *Chemical oceanography*, J. P. Riley and G. Skirrow, eds. Academic Press, London.
- Chevremont, M., and H. Firket. 1951. Action of beryllium on cells cultivated *in vitro*; effects on mitosis. *Nature* **167**: 772.
- Delaney, M. L. 1983. Foraminiferal trace elements: uptake, diagenesis, and 100 M.Y. paleochemical history. Ph.D. thesis. 253 pp. Massachusetts Institute of Technology and the Woods Hole Oceanographic Institution.
- Dodd, J. R. 1967. Magnesium and strontium in calcareous skeletons: a review. *J. Paleontol.* **41**: 1313–1329.
- Gallager, S. M., J. P. Bidwell, and A. M. Kuzirian. 1989. Strontium is required in artificial seawater for embryonic shell formation in two species of bivalve molluscs. Pp. 349–366 in *Origin, History and Modern Aspects of Biomineralization in Plants and Animals*, R. Crick, ed. Plenum Press, New York.
- Hanlon, R. T., J. P. Bidwell, and R. Tait. 1989. Strontium is required for statolith development and thus normal swimming behaviour of hatching cephalopods. *J. Exp. Biol.* **141**: 187–195.
- Kinsman, D. D. J. 1969. Interpretation of Sr^{+2} concentrations in carbonate minerals and rocks. *J. Sediment. Petrol.* **39**: 373–392.
- Krampitz, G., and G. Graser. 1988. Molecular mechanisms of biomineralization in the formation of calcified shells. *Agnew. Chem. Int. Ed. Engl.* **27**: 1145–1156.
- Lev, R., and S. S. Spicer. 1964. Specific staining of sulphate groups with alcian blue at low pH. *J. Histochem. Cytochem.* **12**: 309.
- Linde, A. 1981. On enzymes associated with biological calcification. Pp. 559–570 in *The Chemistry and Biology of Mineralized Connective Tissues*, A. Veis, ed., Elsevier North Holland, Inc.
- Marks, S. C., Jr., and S. N. Popoff. 1988. Bone cell biology: the regulation of development, structure, and function in the skeleton. *Am. J. Anat.* **183**: 1–44.
- Mel'ester, M. E., D. F. Martin, and W. H. Taft. 1970. Effects of alkaline-earth metal ions on the transformation of aragonite to calcite in aqueous solution. *J. Inorg Nucl. Chem.* **32**: 391–399.
- Muller, L. L., and T. J. Jacks. 1975. Rapid chemical dehydration of samples for electron microscopic examinations. *J. Histochem. Cytochem.* **23**: 107–110.
- O'Day, D. H. 1972. An analysis of the effects of cyclic AMP and beryllium on alkaline phosphatase activity during the development of the cellular slime mold. Ph.D. Thesis, University of Delaware. 86 pp.
- Raven, C. P. 1966. *Morphogenesis: the Analysis of Molluscan Development*. Pergamon Press, New York. 366 pp.
- Spicer, S. S., and D. B. Meyer. 1960. Histochemical differentiation of acid mucopolysaccharides by means of combined aldehyde fuchsin-alcian blue staining. *Am. J. Clin. Path.* **33**: 453–460.
- Vittur, F., N. Stagni, L. Moro, and B. de Bernard. 1984. Alkaline phosphatase binds to collagen: a hypothesis on the mechanism of extracellular mineralization in epiphyseal cartilage. *Experientia* **40**: 836–837.
- Yoshioka, S., S. Ohde, Y. Kitano, and N. Kanamori. 1986. Behavior of magnesium and strontium during the transformation of coral aragonite to calcite in aquatic environments. *Mar. Chem.* **18**: 35–48.